# Advances in Microarray Hybridizations at NIEHS

Sherry Grissom

Microarray User's Meeting

June 21, 2002

# Evolution of the Microarray Lab

| Where We Were | Where We Are | Why It's Good |
|---|---|---|
| 200 µg RNA needed per microarray chip | 100-200 µg RNA/ microarray experiment (4 chips) | DIR likes us better! |
| Microcon cleanup | Qiagen cleanup | Time, clean chips |
| 96-well PCRs by hand | Qiagen BioRobot | Saves time |
| Single Manual Sequencing | Qiagen BioRobot (96-well) | Saves time |
| 4 & 8 pin printing | 32 pin printing | Saves time |
| Axon Scanner | Agilent Scanner | Time to do other things, not subjective |
| ArraySuite 1.3 | ArraySuite 2.0 | Quality & Signal-to-Noise Cutoffs |

# Indirect vs. Direct Labeling

| Protocol | μg RNA | Method | Time |
|---|---|---|---|
| **NIEHS** | 25–50 | Direct incorporation of dTTP analogs Cy3-dUTP & Cy5-dUTP at RT step | 5-6 hours |
| **3DNA (Genisphere)** | 1 | Indirect, Primer oligo with capture sequence attaches at dT tail, bind capture reagent that recognizes capture sequence | 5-6 hours |
| **Atlas Glass (Clontech)** | 10-20 | Indirect RT incorporation of aminoallyl-dUTP, Amine reactive dye added. | 8 hours |

Cy3 mean=1881
Background=265

Cy5 mean=1551
Background=441

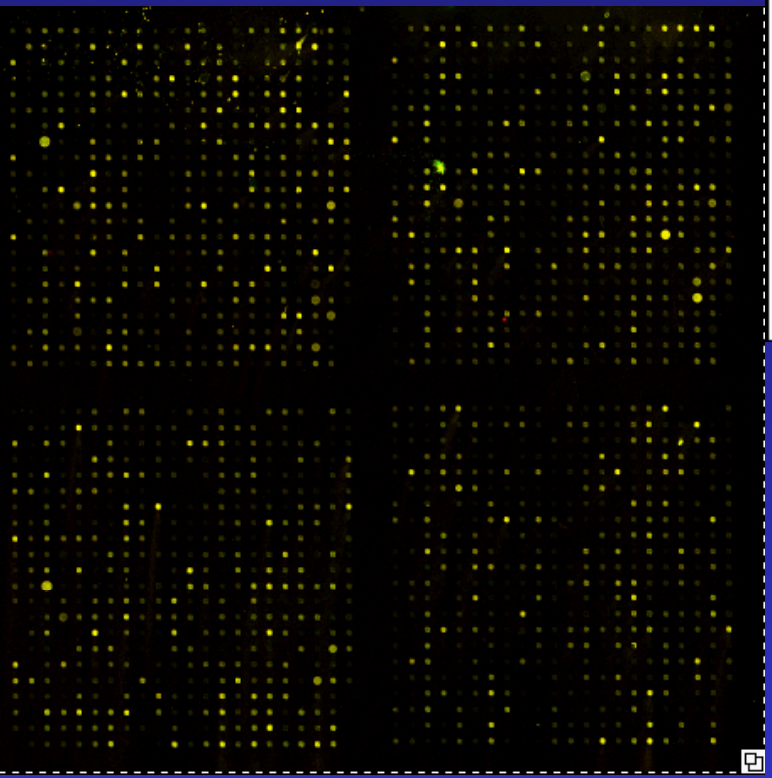
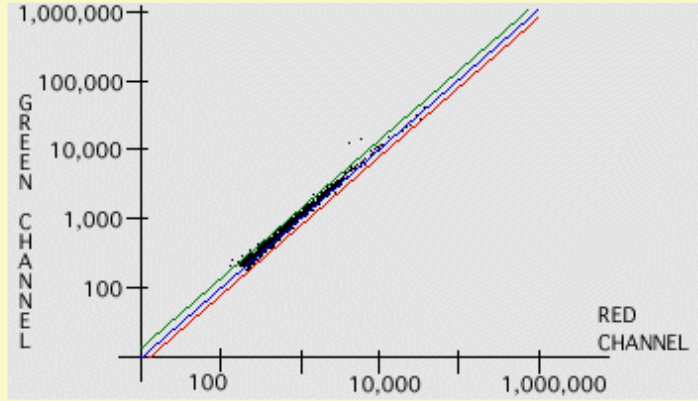


Image Name: p54s75_635_flpmrk.ip
p54s75_532_flp.ip

Histogram / Scatter Plot




Data from:
All targets
Control targets

Calibration by:
Internal Controls
All targets
Background

Calibration Method:
Ratio Distribution
Log-Normal

Log Scale
Calibrated Result

| | | | |
|---|---|---|---|
| Confidence Level: | 99.00 | Intensity From: | 200 |
| Ratio Lower Limit: | 0.76 | Intensity To: | 65535 |
| Ratio Upper Limit: | 1.32 | Target Size From: | 100 |

Refine Stats

CV = 0.075
M = 0.901

Default Setting | Ratio Outliers
Update dataSheet | Equal Intensities

Exit | Refresh

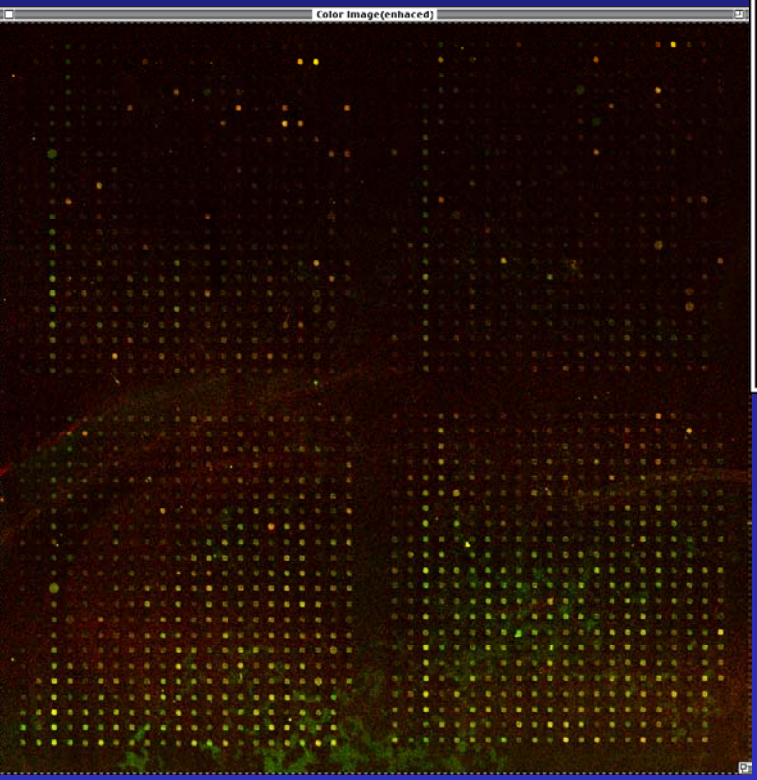
TargetLocator, 1.3, © 1997, NIH/NHGRI/LCG

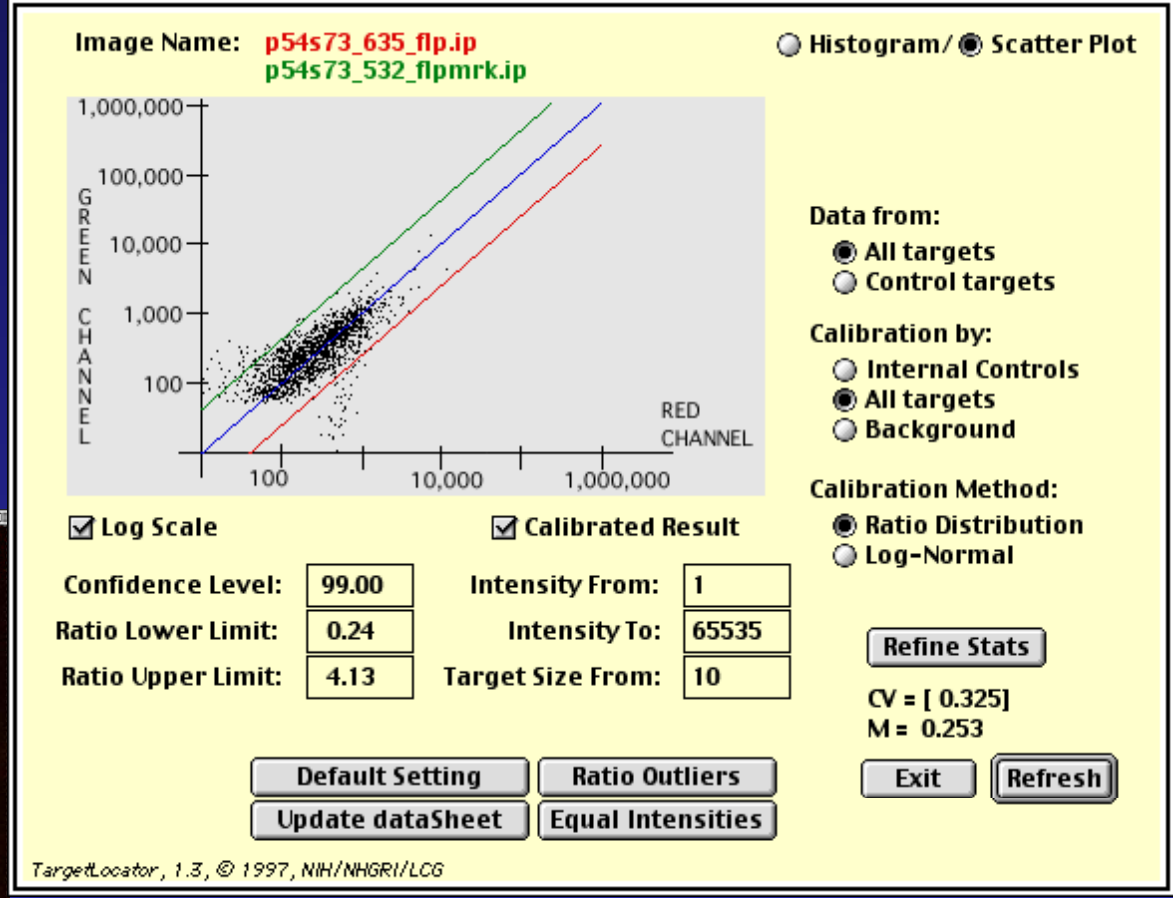
- Very tight scatterplot with few outliers
- Low variation and M=1 reflect a balance between channels.

# Direct Labeling by NIEHS SOP

Cy3 mean=303
Background=101

Cy5 mean=93
Background=80




- Poor signal-to-noise ratio.
- Wide scatterplot reflects poor dye incorporation.
- High CV and M value reflect imbalance.

# 3DNA by Genisphere

# Product Evaluations

- We have also looked at Molecular Probes ARES, Stratagene Fair Play, other reverse transcriptases and Agilent Human 2 cDNA microarrays
- At this time, our current direct labeling protocol is best for in-house arrays.
- Genisphere 3DNA kit is constantly being updated, will try again.
- Soon will retry Clontech's Atlas Array protocol (been tried before with promising results) and Invitrogen's Fluoroscript labeling kit.

# How the Agilent Bioanalyzer has made my life a bit easier and how it can help yours

# Utilities of the Agilent Bioanalyzer


RNA Quality Measure: qualitative tool to measure RNA integrity for labeling.

- Degradation of either 18S or 28S rRNA
- Concentration
- Sometimes, genomic DNA contamination

# Bioanalyzer Test Submissions

**Bioanalyzer Test Submission Form**

*This form must be included with all samples submitted to the NIEHS Microarray Center for test on the Agilent Bioanalyzer.*

Name and Contact Information (room #, phone #):

Email Address:

Principal Investigator:

Project (proposal) Title:

**Sample Submission Requirements**
**(samples will be returned if all criteria are not met):**
*Please check boxes when criteria are met*

☐ a. RNA concentration of a 100-200 ng/ul RNA in nuclease-free water.

☐ b. Minimum volume of 5 ul in an autoclaved tube, delivered on dry ice.

☐ c. If two or more samples are isolated with different procedures/protocols please indicate the differences (this could help us give better advice in the future).

☐ d. Did someone in the Microarray Center give any advice/help on your isolation, if so, please indicate this and the member of the Center who did.

***Please** provide unambiguous names for RNA (no 3-letter codes or numbering 1-12). If we find that the annotations given to separate samples are confusing, we will return this form for further clarification.

****Please** try to limit test samples to no more than 12 at a time.

*****Because** they are test samples, RNA samples submitted in this way are **not** considered a priority for the Bioanalyzer. They will be ran as soon as space is available.

For Bioanalyzer test sample submission, please contact **Sherry Grissom** either by phone (*x0747*) or email (**grissom2**).

| RNA INFORMATION | | | | | |
|---|---|---|---|---|---|
| Tube # | RNA Name | RNA Type (tissue, cell line) | RNA Species (total, mRNA) | Prep Date | Prep Procedure |
| 1 | | | | | |
| 2 | | | | | |
| 3 | | | | | |
| 4 | | | | | |
| 5 | | | | | |
| 6 | | | | | |
| 7 | | | | | |
| 8 | | | | | |
| 9 | | | | | |
| 10 | | | | | |
| 11 | | | | | |
| 12 | | | | | |

http://dir.niehs.nih.gov/microarray/methods/bioanalyzer.pdf

# Utilities of the Agilent Bioanalyzer


Labeled RNA Quality Measure: qualitative tool to measure labeled RNA integrity for hybridization.

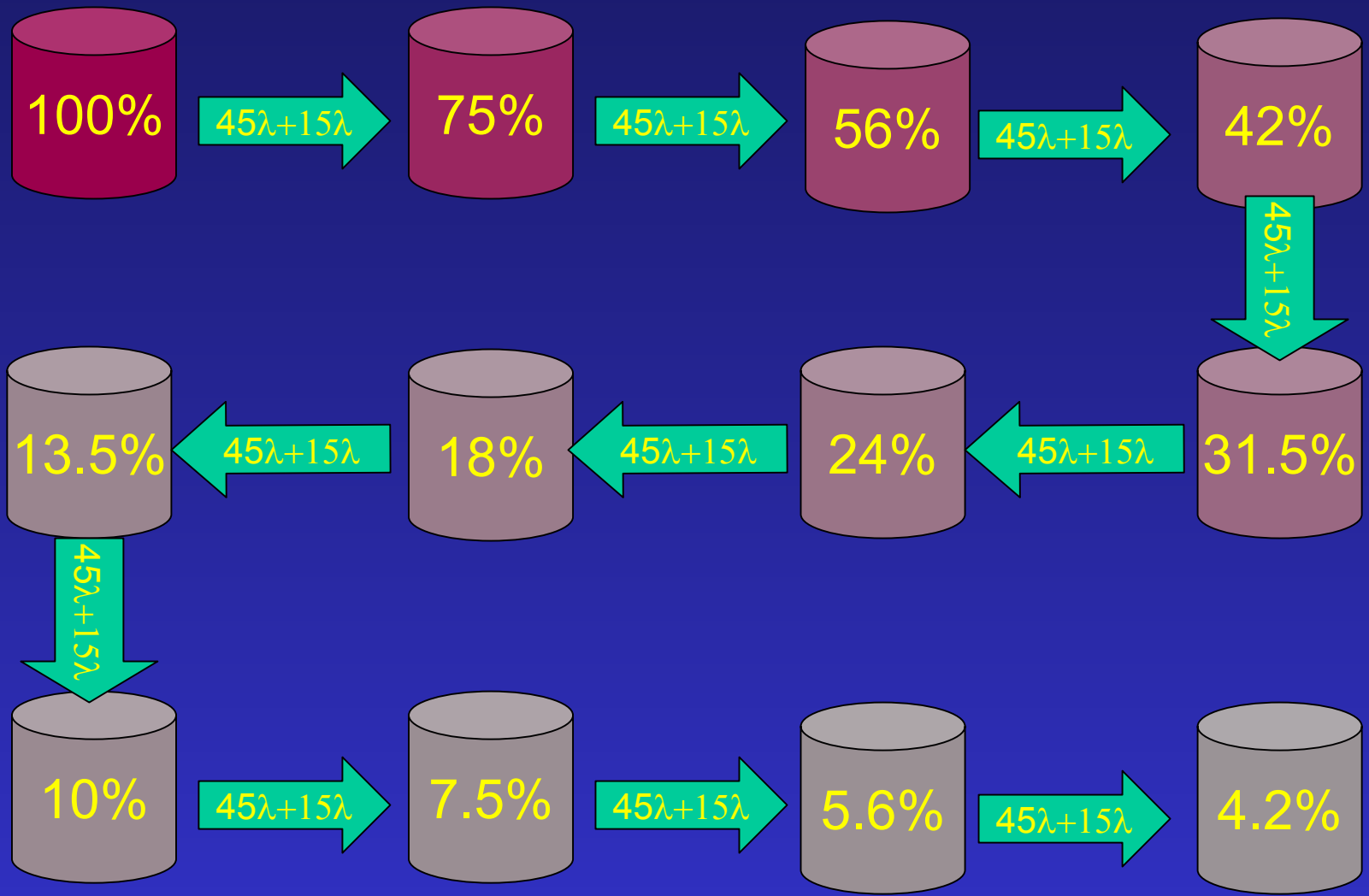
- Is there a problem with the labeling and reverse transcription reaction?
- "Cheaper" way to optimize protocol
- Others use spectrophotometer and mini-gels to test the integrity of their labeled probe.

# Spectrophotometer vs. Agilent Bioanalyzer

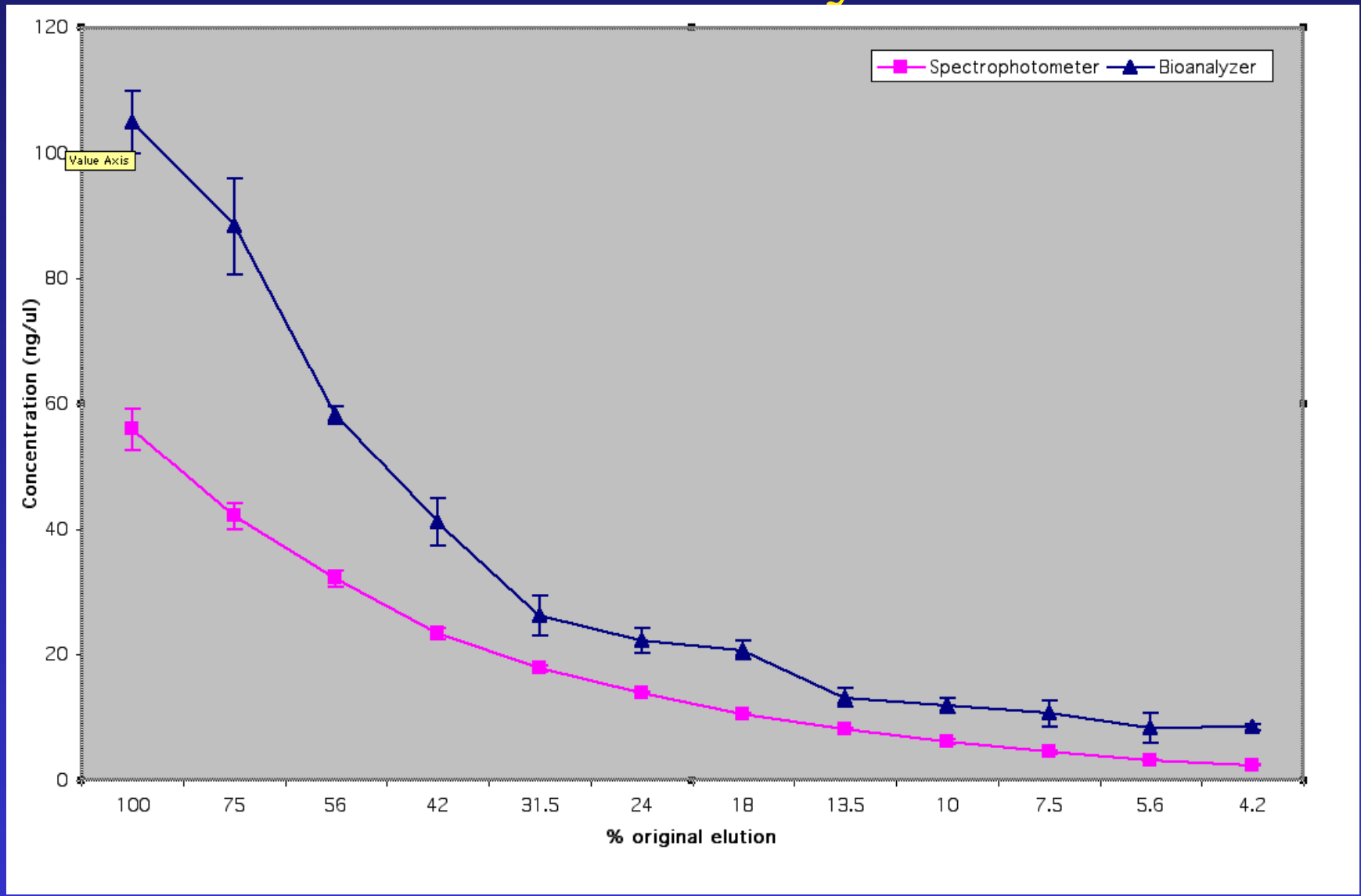
- Spectrophotometer output is absorbance at a given wavelength.
- From the absorbance measurements, can calculate concentration, labeling density, and frequency of dye incorporation.
- No way to tell about dye carry over.
- Need at least 50 µL sample.

- Bioanalyer output is in form of an electropherogram.
- Directly outputs **semi-quantitative** labeled RNA concentrations and unincorporated Cy5 amounts.
- Gives relative idea of amount of dye being carried over.
- Only need 1µL of sample

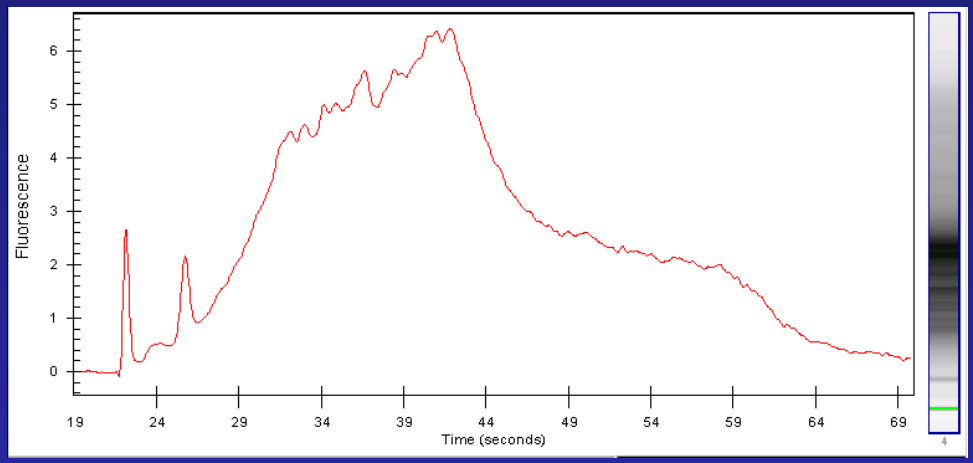
# Dilution Scheme



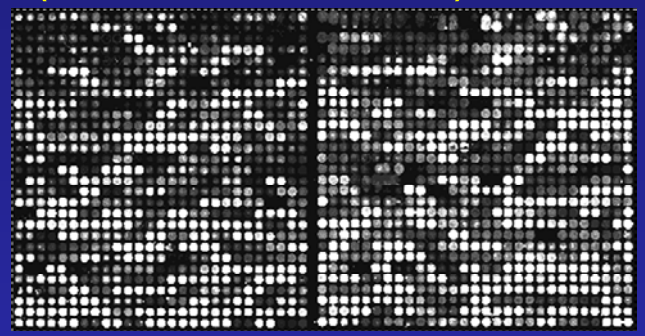
# Comparison of Spectrophotometer and Bioanalyzer

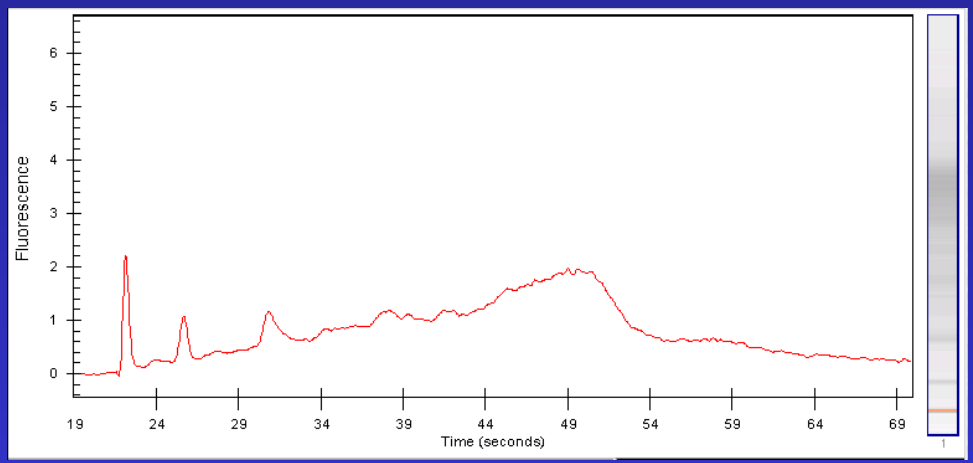


# Cy5 Incorporation as a Measure of RT reaction efficiency

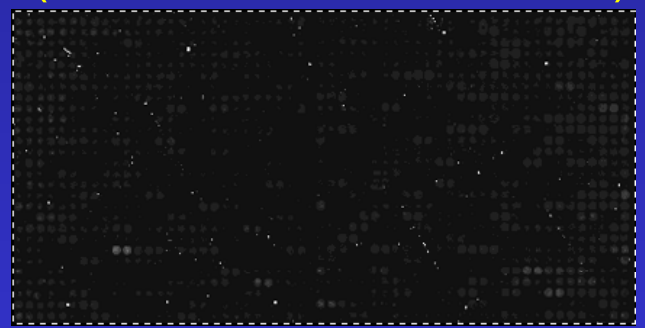



NMC's QC RNA
(we know it labels)






Questionable RNA
(we know it doesn't label)



# Summer Exp. 1- Negative Control Background Subtraction

- Should we be doing global or local background subtraction? Currently, we are assuming that what is "beside" a spot is equal to what is "underneath" that spot. Is this a correct assumption?
- Print a rat chip, replace 566 spots on the array (17 per sub-array) with negative control cDNA spots (Arabidopsis Cab), perform hybridizations. Data analysis performed by Terry Speed at UC-Berkeley.
- Theoretically, there should be no signal obtained from this spot because there will be no cross-hybridization. In practice, signal will be obtained from these spots as a result of cross-hybridization and autofluorescence.

# Summer Exp. 2 -Qualitymetrics

- Artificially derived sequences from yeast intergenic regions (YIRs) with no cross-reactivity among species.
- Spike-In = YIR cDNA spotted and corresponding mRNA sequence of known concentration "spiked" into reverse transcription reaction.
  - Calibration controls are used to determine the dynamic range of the reaction.
  - Ratio controls tell us how well each of the labeling reactions went.
- Negative Controls
  - No cross-hybridization (should be the same as no spot)
  - How do they compare to buffer blanks?
  - Is there uniform distribution across the chip?
- Positive Controls
  - Was the label a success?
  - Is there uniform distribution across the chip?

# A few intriguing questions for later…

- How low can you go? Getting the maximum amount of signal for the minimum quantities of RNA and other reagents.

- How can we measure genomic contamination, why is it detrimental to the microarray process and what can we do about it?

# Future Goals

- Bioanalyzer for measuring Cy5 incorporation to:
  - Can we use less RNA and get comparable signals?
  - Optimize RT enzyme for time and temperature.
  - Optimize Cy dye concentrations to reflect lower amounts of RNA used for hybridizations.
- Continue DIR projects (since August 2001, I have performed ~400 hybridizations).

# Thanks

- Jeff Tucker
- Cindy Afshari
- Rick Paules
- Astrid Haugen
- Danica Ducharme
- Valerie Moorman
- Neysa Garner

For questions, please visit http://dir.niehs.nih.gov/microarray or D226